DOI 10.1002/pmic.200300466 *Proteomics* 2003, *3*, 1988–2001

Application of laser capture microdissection combined with two-dimensional electrophoresis for the discovery of differentially regulated proteins in pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is the most lethal of all the common malignancies and markers for early detection or targets for treatment of this disease are urgently required. The disease is characterised by a strong stromal response, with cancer cells usually representing a relatively small proportion of the cells in the tumor mass. We therefore performed laser capture microdissection (LCM) to enrich for both normal and malignant pancreatic ductal epithelial cells. Proteins extracted from these cells were then separated by two-dimensional gel electrophoresis (2-DE). The limited amounts of protein in the LCM procured samples necessitated the detection of 2-DE resolved proteins by silver staining. Consequently, loading equivalent amounts of protein onto gels was essential. However, we found that conventional means of measuring total protein in the samples were not sufficiently accurate. We therefore adopted a strategy in which the samples were first separated by one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis, stained with silver stain and subjected to densitometry. Evaluation of the staining intensity was then used to normalise the samples. We found that the protein profiles from undissected normal pancreas and LCM-acquired nonmalignant ductal epithelial cells from the same tissue block were different, underpinning the value of LCM in our analysis. The comparisons of protein profiles from nonmalignant and malignant ductal epithelial cells revealed nine protein spots that were consistently differentially regulated. Five of these proteins showed increased expression in tumor cells while four showed diminished expression in these cells. One of the proteins displaying enhanced expression in tumor cells was identified as the calcium-binding protein, S100A6. To determine the incidence of S100A6 overexpression in pancreatic cancer, we carried out immunohistochemical analysis on sections from a pancreas cancer tissue array containing 174 duplicate normal and malignant pancreatic tissue samples, from 46 pancreas cancer patients. Normal pancreatic ductal epithelia were either devoid of detectable S100A6 or showed weak expression only. Moderately or poorly differentiated tumors, by contrast, showed a higher incidence and a higher level of S100A6 expression. These observations indicate that the combination of LCM with 2-DE provides an effective strategy to discover proteins that are differentially expressed in PDAC.

Keywords: Differential protein expression / Laser capture microdissection / Pancreatic cancer
PRO 0466

1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer deaths in the Western world, accounting for 40 000 deaths *per* year in Europe and 28 000 deaths *per* year in the USA [1, 2]. Outside of very select groups (familial cancer syndromes and chronic and hereditary pancreatitis) there is currently no valid approach for screening for the disease [3] and the cancer is almost invariably advanced when diagnosed [4, 5]. The overall long-term survival is consequently bleak at 0.4% to < 5% [6, 7]. Earlier diagnosis and new treatments are therefore desperately needed for PDAC.

Proteomic technologies, including high resolution two-dimensional electrophoresis (2-DE), and advances in mass spectrometry, are providing new opportunities for

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Abbreviations: LCM, laser capture microdissection; **LDH**, lactate dehydrogenase; **PDAC**, pancreatic ductal adenocarcinoma

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uncovering markers and/or therapeutic targets for cancer [8, 9] and should be explored for pancreatic cancer. Moreover, the identification of proteins whose expression is altered in pancreatic cancer has the potential to reveal important mechanisms underlying this disease.

A major obstacle, however, to the analysis of pancreatic cancer specimens is the degree of tissue heterogeneity. The fully malignant stage of pancreatic cancer almost invariably elicits a vigorous, non-neoplastic stromal response with cancer cells representing on average, 25% of the cells in the tumor [10]. Moreover, normal ductal epithelial cells, from which the cancer is believed to arise, represent as little as 5% of the normal pancreas. Various approaches have been employed recently in order to obtain cell populations enriched for nonmalignant or malignant pancreatic ductal cells for the purposes of differential gene expression analyses [11–14]. These have included short-term cultures of pancreatic ductal epithelial cells [11, 12], fine needle aspiration of cancer cells [13] and laser capture microdissection (LCM) [14]. Each of these approaches has its merits and its disadvantages. Culturing cells, in effect, increases the quantity of sample material, which can be limited. However, even short-term culture may result in unavoidable changes in protein expression [15, 16]. Fine needle aspiration of cancer cells is efficient, however control nonmalignant cells cannot be acquired in the same manner. Finally, while LCM effectively provides enriched populations of target cells, it requires a considerable commitment of time and is labor intensive.

Although previous studies have reported changes in mRNA levels that accompany PDAC [11–14], a proteomic approach to the analysis of protein expression in primary pancreas tissue samples has not been reported. We have therefore examined protein expression in nonmalignant and in malignant pancreatic ductal epithelia. Samples were obtained from primary human biopsy material and LCM was used to enrich for ductal epithelial cells. Proteins extracted from these cells were resolved by 2-DE. Research involving other cancers, including cervical [17], prostate [18], breast [19], ovarian [20] and oesophageal [21] has demonstrated the compatibility of LCM with 2-DE.

We show that frozen pancreas specimens are amenable to proteomic analysis. We demonstrate that enrichment of the ductal epithelial cells by LCM facilitated the direct comparison between the protein profiles of the normal and tumor cell type. Our analyses lead to the detection of five proteins that were up-regulated in tumor cells and four that were down-regulated in these cells. Among those up-regulated, we identified annexin III and S100A6. Lactate dehydrogenase (LDH) and trypsin, by contrast, were more abundant in normal ductal epithelia than in tumor cells. We examined the up-regulation of S100A6 in pancreas cancer cells by immunohistochemistry and demonstrated that moderately or poorly differentiated pancreas tumors contain high levels of S100A6 compared with their normal counterparts. Our observations suggest that the combination of LCM with 2-DE and mass spectrometry can be used for the detection of proteins that are differentially expressed between normal and malignant pancreatic ductal epithelial cells.

2 Materials and methods

2.1 Tissue preparation and staining

Pancreatectomy specimens were obtained following surgery, with full ethical consent, and examined by a specialist pathologist. Areas of tissue, considered upon macroscopic viewing to contain malignant or nonmalignant pancreas were selected, cryofixed in liquid isopentane (−160°C) cooled by liquid nitrogen, and stored at −80°C. Occasionally, samples were obtained that were preserved by snap freezing in liquid nitrogen prior to storage in liquid nitrogen. For microscopic examination, 5 μm thick frozen sections were cut onto slides, stained with haematoxylin and eosin (H & E), as described below and examined by a pathologist. Only samples with a histological diagnosis of PDAC were included in the study. Of necessity, samples with few ductal epithelial cells in the nonmalignant component were excluded. For staining, 7 μm thick frozen sections were cut onto slides (that were precleaned using detergent, washed with deionised water and oven-dried at 40°C) using a Bright (Huntingdon, Cambs, UK) OTF 5000 cryostat (chamber temperature −25°C). Sections were placed on dry ice or kept in the cryostat chamber prior to staining.

H & E staining was carried out only for monitoring of tissue sections and was not used in conjunction with LCM. Sections were fixed (70% ethanol for 1 min), H & E stained (Ehrlich's haematoxylin for 30 s, Scott tap water for 10 s, eosin for 10 s) and dehydrated (70% ethanol for 30 s, followed by 3 × 100% ethanol eosin for 10 s, xylene 2 × for 5 min). Methyl green staining of the sections used for LCM was as follows: sections were fixed (70% ethanol for 1 min), washed in deionised water for 15–30 s, stained with violet-free methyl green (2% w/v in deionised water) for 30 s, rinsed twice in deionised water and dehydrated (70% ethanol for 30 s, 95% ethanol for 30 s, 2 × 100% ethanol for 30 s, xylene 2 × for 5 min). Complete protease inhibitor cocktail tablets (Roche) were added to the staining solutions for both protocols (one tablet/80 mL solution).

2.2 Laser capture microdissection

Following staining with methyl green, sections were air-dried and microdissected using an Arcturus PixCell II system (Arcturus, Mountain View, CA, USA) with a 7.5 μm laser beam. Power and pulse duration were adjusted as required during microdissection. These were typically 70 mW and 3–10 ms respectively. Following optimization of conditions, an estimated 50 000 cells were laser captured using an average of 25 000 pulses and the times for LCM of normal and tumor ductal cells were approximately 14 h and 4 h, respectively. Microdissection caps were inserted into 0.5 mL microcentrifuge tubes containing 50 μL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base) and the cells solubilized by inversion of the tubes for 30 min at room temperature, followed by vortex-mixing for 15 s and a brief pulse-centrifugation at 12 000 *g*. Tissue from multiple caps was extracted into the same aliquot of lysis buffer until sufficient material had been collected. Where necessary, tubes were stored at −80°C until further use. In total, five or six tubes, each containing 50 μL of lysis buffer were used for each specimen, with the lysates from each tube being pooled.

2.3 Estimation of sample protein concentrations relative to a reference sample lysate

A protein lysate, prepared from an undissected pancreas tumor specimen, was passed through a 21 gauge needle to reduce its viscosity, ultracentrifuged and the supernatant aliquoted and stored at −80°C. This lysate was then tested, by separating increasing quantities of it in two dimensions, as described below, to determine the quantity required to produce a high quality silver-stained gel image with an appropriate number of protein features (~800 spots). This quantity of lysate was then used as a reference against which relative sample protein concentrations of LCM procured material were estimated. To do this, two-fold dilution series, from undiluted to a 1-in-16 dilution, of the test samples and reference sample were prepared in lysis buffer. All samples were then mixed with two volumes of sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 25% w/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol) containing a trace of bromophenol blue and heated at 95°C for 10 min. Test and reference samples were then subjected to 1-D SDS-PAGE on small format gels (10% separating and 4% stacking gels) at a constant voltage of 150 V. Gels were silver stained as described in Section 2.5 with the exception that 1% acetic acid was used to stop development, and no further washing was carried out. Gels were densitometrically scanned and analysis using TotalLab software (NonLinear Dynamics, Newcastle upon Tyne, UK) was used to inform our estimation of relative protein amounts in the test samples compared to the reference sample.

2.4 IEF and second dimension SDS-PAGE

Samples acquired either from laser captured cells or from lysates of whole pancreatic sections were clarified by centrifugation at 100 000 *g* for 30 min at 4°C. Supernatants (250 μL) were mixed with 120 μL of a sample loading solution (9 M urea, 2% CHAPS and a trace of bromophenol blue). IPG buffer (containing carrier ampholytes, pH range 3–10 nonlinear; Amersham Biosciences, Uppsala, Sweden) was added to a final concentration of 2% v/v. IPG focusing strips (18 cm in length, pH range 3–10, nonlinear; Amersham Biosciences) were passively rehydrated overnight at room temperature with 350 μL of sample mix. IEF was carried out on a Multiphor II system (Amersham Biosciences), with an initial linear gradient of 0–500 V over 1 min, then 500–3500 V linearly over 1.5 h, followed by 3500 V for 5 h 40 min. Prior to the second dimension separation, strips were equilibrated in two successive buffers, each containing 1.5 M Tris-HCl (pH 8.8), 6 M urea, 34.5% v/v glycerol, 2% w/v SDS and a trace of bromophenol blue. The first buffer also contained 1% w/v DTT, while the second contained 2.5% w/v iodoacetamide. Each equilibration was for 15 min with continuous agitation with 10 mL of buffer *per* IPG gel. Strips were then rinsed in electrophoresis buffer (25 mM Tris base, 192 mM glycine and 0.1% w/v SDS), applied to 12% acrylamide gels and sealed with melted agarose (0.5% w/v agarose in electrophoresis buffer containing a trace of bromophenol blue). Electrophoresis was carried out using an Ettan Dalt II apparatus (Amersham Biosciences), with initial separation at a constant 5 W *per* gel for 30 min followed by 20 W *per* gel until the dye front had migrated approximately 18 cm at 25°C. The total run time was typically 4–4.5 h. Once electrophoresed, gels were transferred to polypropylene containers and immersed in fixative (40% methanol, 7% acetic acid, in water), prior to staining.

2.5 Silver staining of polyacrylamide gels

Silver staining was performed as described by Yan *et al.* [22]. Gentle agitation of the gels on a rotary platform was used throughout. Gels were fixed in 10% acetic acid, 40% ethanol, before sensitization for 30 min in a buffer containing 30% v/v ethanol, 0.2% sodium thiosulphate and 0.83 M sodium acetate. Gels were then subjected to 3 × 5 min washes with water and subsequently equilibrated with 0.25% w/v silver nitrate for 20 min, rinsed twice in water and developed in 2.5% w/v sodium carbonate containing 0.04% v/v formaldehyde (37% solu-

tion). Development was stopped with 40 mM EDTA and gels were washed three times in water, scanned as described in Section 2.8 and stored at 4°C.

2.6 Colloidal Coomassie blue staining of polyacrylamide gels

Coomassie blue staining was performed according to Neuhoff *et al.* [23]. Briefly, gels were stained for three hours in a 4:1 mixture of staining solution (containing 0.1% w/v Coomassie brilliant blue G250 dye, 2% orthophosphoric acid and 10% w/v ammonium sulphate) and methanol. Gels were destained briefly in a solution containing 10% v/v acetic acid, 25% v/v methanol, and subsequentiy stored in 25% methanol at 4°C.

2.7 Identification of proteins by MS

Protein spots of interest were excised from Coomassie stained gels and trypsin digested essentially according to the method of Courchesne and Patterson [24]. Briefly, the excised gel spots were washed in 50% v/v acetonitrile/25 mM w/v ammonium bicarbonate, pH 7.8, and dried in a SpeedVac. The dried gel spots were rehydrated with 4–10 μL digestion buffer (10 μg/mL modified sequencing grade trypsin in 25 mM NH_4HCO_3) and incubated overnight at 37°C. The resulting peptides were extracted by the addition of 4 μL water followed by 7 μL 30% v/v acetonitrile/0.1% v/v TFA with mixing and brief centrifugation. The supernatants were recovered and mixed 1:1 with matrix (10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile/50% v/v ethanol/0.001% v/v TFA) containing adrenocorticotropic hormone (ACTH, 50 fmol/μL) and 1 μL of the mixture was spotted onto a 96-position stainless steel target. Peptide mass fingerprints were obtained semiautomatically on a MALDI-TOF mass spectrometer (Micromass, Manchester, UK) and resultant mass lists searched against a nonredundant protein database (SWISS-PROT/TrEMBL) using ProteinLynx 3.4 software (Micromass). Mass lists were subsequently used to search the NCBI database using MASCOT software (Matrix Science, London UK; URL:http://www.matrixscience.com).

In some cases the scores obtained for the identification of proteins were not sufficiently high to provide a reliable identification. In these cases the digests were subsequently analyzed by MALDI-TOF/TOF (4700, Applied Biosystems, Foster City, CA, USA) which comprises a MALDI source with a 200 Hz frequency tripled neodinium yttrium-aluminium-garnet laser operating at 355 nm wavelength. For MS mode the ions were accelerated at 20 kV through a grid at 14 kV into the first, linear, field-free drift region, through the empty, turned-off collision cell, through the second source (where they do not experience any re-acceleration) through the second, linear, field-free drift region, into the reflector and thence to a microchannel plate detector. For MS/MS the ions were accelerated at 8 kV through a grid operating at approximately 6.7 kV into the first, linear, field-free drift region. In this region the ions passed through a timed-ion-selector device which was used to select one ion from the mixture of ions of different mass. The selected ions were subsequently passed through a retarding lens where they were decelerated into the collision cell (at 1 kV with air at a pressure of 1×10^{-6} Torr). After passing through the collision cell the ions were re-accelerated in the second source region at 14 kV, passed through the second, field-free, linear drift region, into the reflector and finally to the detector.

2.8 Scanning and analysis of gels

Gel images were obtained using a GS-710 scanner, at 300 dpi resolution using the green channel for silver-stained gels and the red channel for Coomassie blue-stained gels (Bio-Rad, Hercules, CA, USA). Data were acquired using PDQuest software (Bio-Rad, V6.2). Image analysis, in particular to determine differences between gels displaying proteins from nonmalignant and malignant cells, was initially carried out manually. Digital images of each subregion of the gels were enlarged and examined by three independent evaluators. Differences identified on images were compared against the actual gels to exclude staining and image acquisition artefacts. Images (exported to TIFF format files from PDQuest) were also subjected to automated analysis using Progenesis Workstation software V2002.1 (NonLinear Dynamics).

2.9 Immunohistochemical analysis of pancreas samples

A rabbit antihuman S100A6 antibody, raised against purified recombinant human S100A6 monomer expressed in *Escherichia coli*, was obtained from DAKO (Ely, Cambs, UK). It was used to perform immunohistochemical analysis on paraffin embedded sections from pancreas specimens used in the 2DE-LCM analysis and also on a pancreatic cancer tissue microarray generated at the Liverpool Cancer Research Tissue Bank, Liverpool, UK, using the method described by Kononen *et al.* [25]. The pancreatic cancer tissue microarray contains matched sets of tumor and non-neoplastic pancreatic tissue samples from 41 pancreatic cancer patients treated at the Royal Liverpool University Hospital between 1994 and 2001.

In addition pancreatic cancer tissue samples from five patients are included with no matched non-neoplastic samples. Each sample is present in duplicate, making 174 pancreatic tissue samples in all.

Five micron thick sections of pancreas specimens or the pancreatic cancer tissue microarray were deparaffinized in xylene and then rehydrated through alcohol to distilled water. Antigen retrieval, as recommended by the suppliers of the S100A6 antibody (DAKO) was performed by pressure-cooking the slides in 10 mM EDTA (pH 7.4) for 3 min. Immunohistochemical staining was performed using an automatic staining system (Universal Staining System; DAKO). Slides were incubated for 40 min with anti-S100A6 antibody (0.25 μg/mL, DAKO), rinsed in PBS and the antibody localization visualized by incubating sections with a horse radish peroxidase conjugated secondary antibody for 30 min followed by diaminobenzidine (DAKO) for 10 min. Slides were counterstained with haematoxylin for 5 min, dehydrated with 100% ethanol and xylene and coverslips mounted with DPX mountant (BDH, Poole, Dorset, UK). Negative controls were incubated only with the labelled secondary antibody.

Scoring of the tissue microarray sections was performed by a specialist histopathologist. The information recorded included the types of cells showing S100A6 staining, the intensity of staining (− = negative; + = weak; ++ = intermediate; +++ = strong); and the subcellular location of staining (nuclear, cytoplasmic). Chi square tests and contingency tests were used for statistical analysis and $p < 0.05$ was considered to indicate a significant difference.

3 Results

3.1 Parameters affecting tissue selection and preservation for LCM

We performed LCM in order to enrich for normal and malignant ductal epithelial cells. However, the scarcity of normal ductal epithelial cells in the nonmalignant component of the pancreas was a limiting factor and determined the suitability of samples for the study. Nonmalignant samples that were archived or sampled without effort to select for regions containing macroscopic duct(s) only rarely contained sufficient ductal epithelial cells and greatly reduced the number of matched samples available. Another factor, important to the success of the LCM, was the method of preservation of pancreas samples. Cryofixation of samples in cooled liquid isopentane (see Section 2.1) resulted in high quality preservation of histology compared with that observed in samples that were snap-frozen in liquid nitrogen. The disruption to tissue morphology in some snap-frozen samples precluded accurate laser capture and resulted in the exclusion of these samples from the study.

3.2 Estimation of relative protein concentration using a reference protein lysate and 1-D SDS-PAGE

Our determination of differentially regulated proteins was made on the basis of differences in the intensity of given spots on selected gels. Given the limited dynamic range and linearity of silver staining, it was therefore important that comparisons were made between gels that had similar amounts of loaded protein. The minute quantities of protein in our laser capture samples combined with the incompatibility of the lysis buffer with conventional protein assays rendered accurate protein estimation difficult. We therefore devised an assay that enabled us (1) to ensure that we had sufficient protein in our samples to yield good quality silver-stained gel images for analysis, and (2) to normalize paired samples sets for loading. Firstly, the quantity of a reference sample lysate (see Section 2.3) that was required to produce a high quality silver-stained gel image with approximately 800 protein features was determined. An example of the image obtained is shown in Fig. 1B. Serial dilutions of this reference sample and of laser captured samples were then subjected to 1-D SDS-PAGE and silver stained, as shown in Fig. 1A. Densitometric analysis of entire lanes and individual protein bands was then used to determine the relative amounts of protein present in each sample. For the examples shown in Fig. 1A, it was estimated that the tumor sample was 1.5-fold more concentrated than the normal sample and 2-D gels (shown in Fig. 1C) were loaded accordingly. Gels were stained in parallel and show similar overall spot intensities. This method was applied to three sets of samples (two patient-matched samples (sets A and B) and four unmatched samples (set C), shown in Fig. 3). Since malignant ductal epithelial cells were abundant in tumor samples, the number of normal ductal epithelial cells available for LCM in the nonmalignant samples, was for each set of samples, the determinant of the quantity of protein processed. In the case of one set (Fig. 3, set B) normal ductal epithelial cells were abundant. Thus a greater level of protein was isolated and loaded on gels in this set than for the three other sets of samples.

3.3 The profile of LCM-procured pancreatic ductal epithelial cells differed from that of undissected pancreas

The benefits of LCM in terms of enrichment of proteins have been reported to be tissue-specific [26]. It was therefore necessary to determine whether the protein pro-

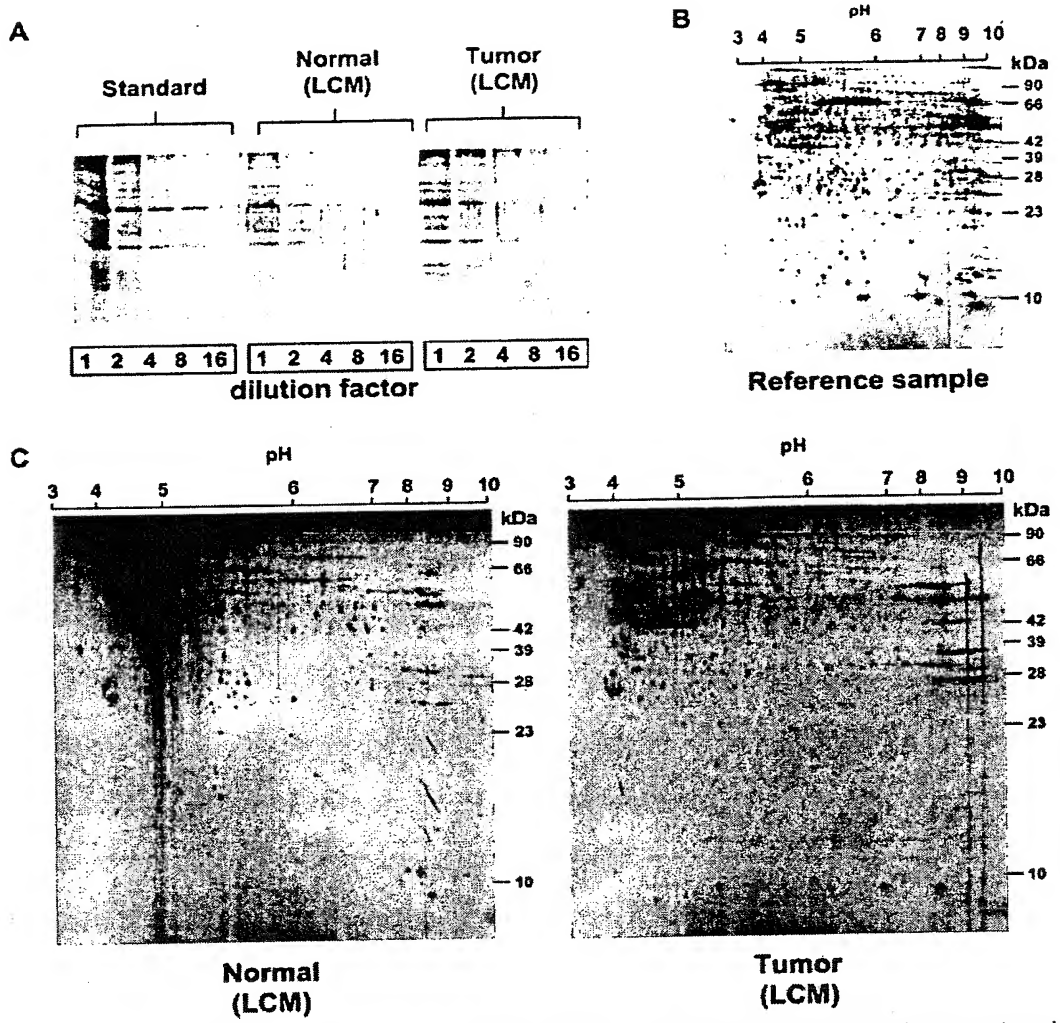

Figure 1. A, 1-D SDS-PAGE gel used to estimate relative protein concentrations in laser captured normal and tumor ductal cell lysates compared to a reference sample lysate. B, A typical silver-stained gel image following 2-D separation of a predetermined (see Section 2.3) quantity of the reference sample. C, Images of laser captured ductal cell protein profiles from lysates following normalisation.

files of undissected normal or undissected malignant pancreas differed from those of dissected normal or dissected malignant pancreatic ductal epithelia. Figure 2 shows the respective profiles obtained using tissue material from a single patient. The protein profile obtained from an undissected cryostat section of nonmalignant pancreas was appreciably different from that of laser capture microdissected nonmalignant ductal cells (Fig. 2, compare upper panels). The inset allows closer inspection of the center regions of these gels. Several differences, some examples of which are highlighted with arrows, are obvious. A comparison of the undissected and dissected tumor samples showed that these profiles were more similar to each other (Fig. 2, compare lower panels) than were the profiles of undissected and dissected normal material. Moreover, normal and malignant dissected ductal epithelial cell profiles (compare upper and lower right panels) were more similar to each other than undissected and dissected normal profiles. Thus, our data support the need to perform LCM to obtain pancreatic ductal epithe-

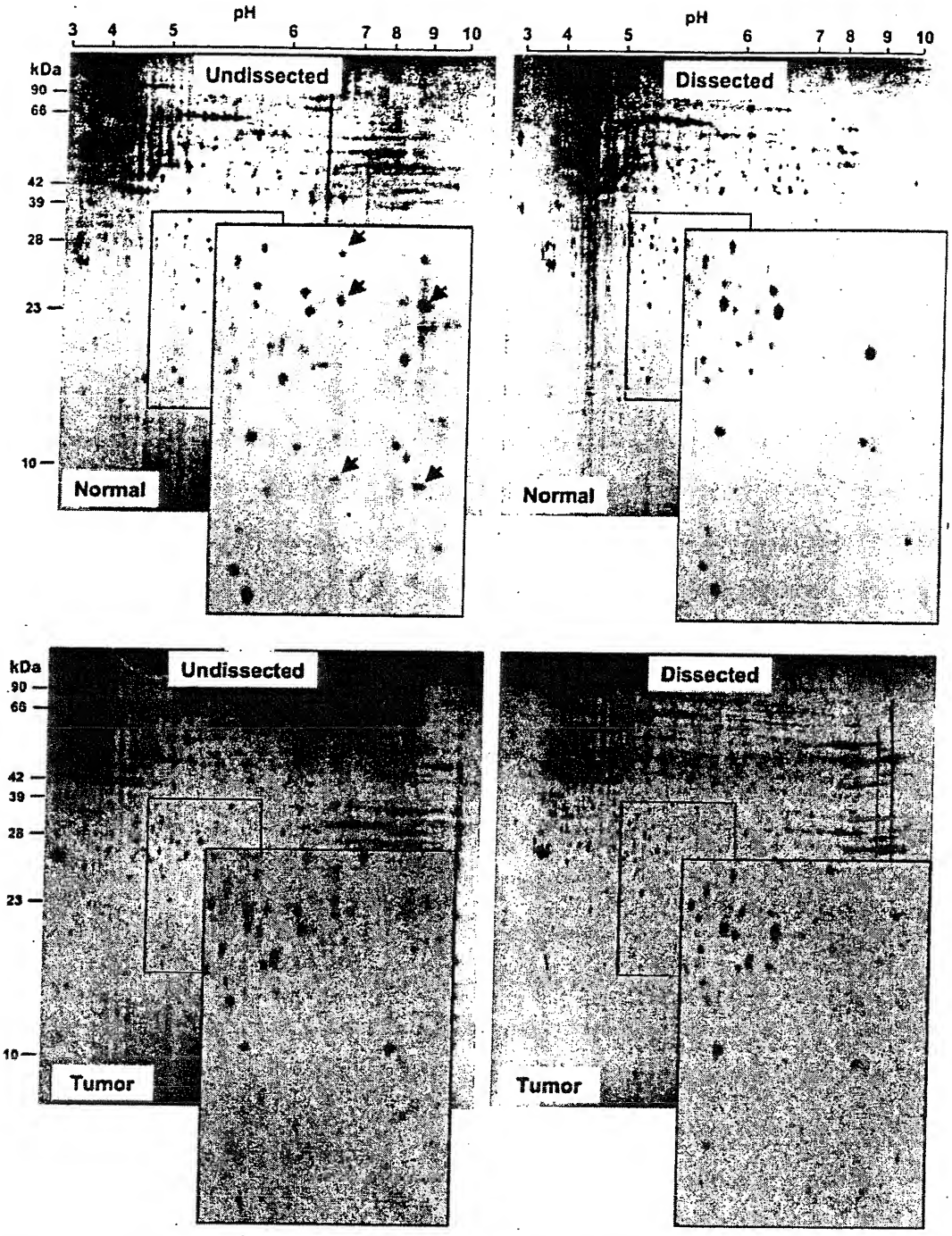

Figure 2. Silver stained gel images from a matched set of normal and malignant pancreatic specimens, where proteins were extracted from undissected tissue or from dissected normal ductal epithelia or dissected malignant cells, as indicated. A similar area of each gel (inset) is magnified and examples of protein spots with substantially different intensities between undissected and dissected normal samples are indicated with arrows.

lial cells, particularly in the case of nonmalignant samples and indicate that LCM will avoid the follow-up of many proteins that are expressed in cells other than ductal cells.

3.4 Normal and malignant pancreatic ductal epithelial cells have similar protein profiles with small numbers of differences

We performed LCM and 2-DE for three sets of samples, two patient-matched sample sets, (Fig. 3, set A and set B) and four unmatched samples (Fig. 3, set C) and examined, manually, each set of gel images for differences in spot intensities. Each sample set contained differences in the intensities of a number of spots. Some of the differences found within a set were not however, apparent in other cases. Nonetheless, analysis of the four nonmalignant and four tumor gels revealed nine protein spots whose intensities varied substantially and consistently between normal and tumor. The locations of these spots are shown in Fig. 3. Five of these spots fall in a small region in the center of the gels and can be seen for each gel in Fig. 3b. Image analysis software (ImageMaster; Amersham Biosciences) allowed the relative spot volu-

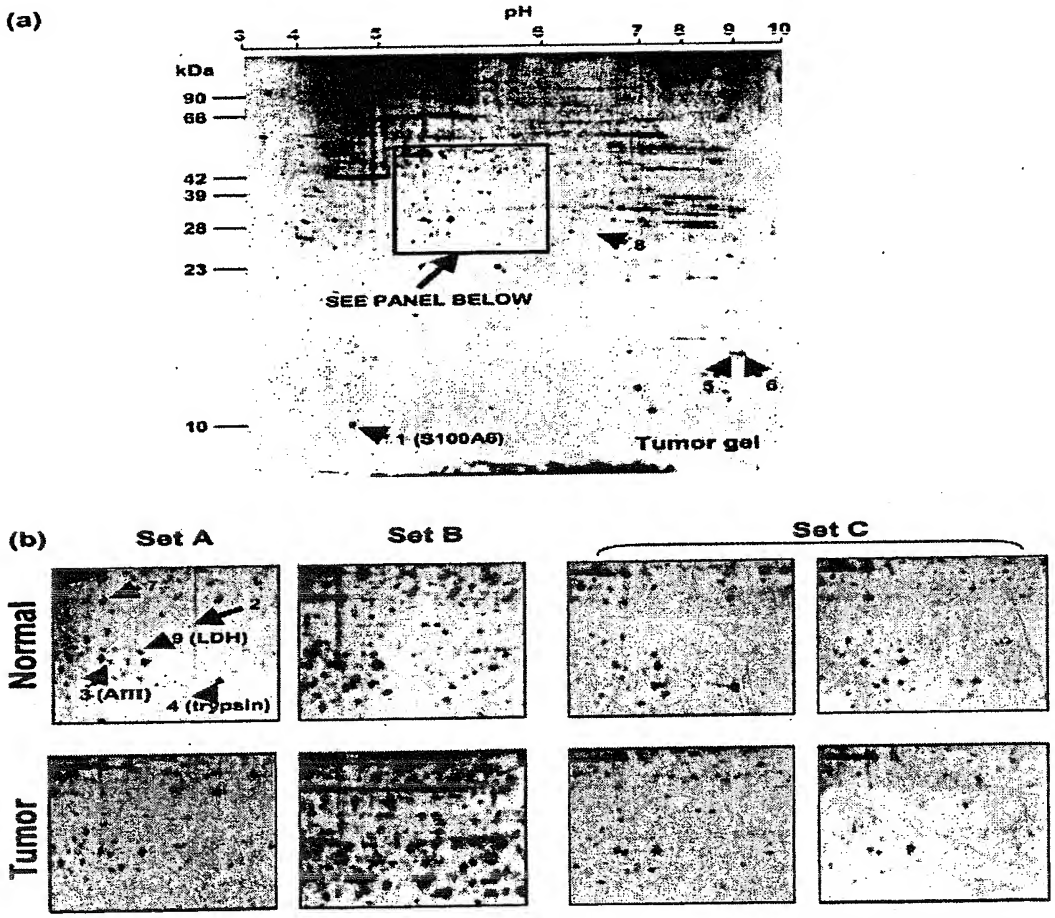

Figure 3. Gel images showing the positions of the nine protein spots identified as differentially regulated. (a) Whole laser captured tumor gel. Note that the arrow for spot 8, down-regulated in tumor and undetected on this particular gel, indicates the approximate expected location of this protein. (b) Images from four dissected normal and four dissected tumor gels show the same part of each gel as indicated in (a). Sets A and B are from patient-matched normal and tumor samples. Set C is from four unmatched patients. Arrows indicate five protein spots that are differentially regulated between normal and tumor samples in all cases. Quantitative analysis for all spots is presented in Fig. 4. AIII = annexin III; LDH = lactate dehydrogenase.

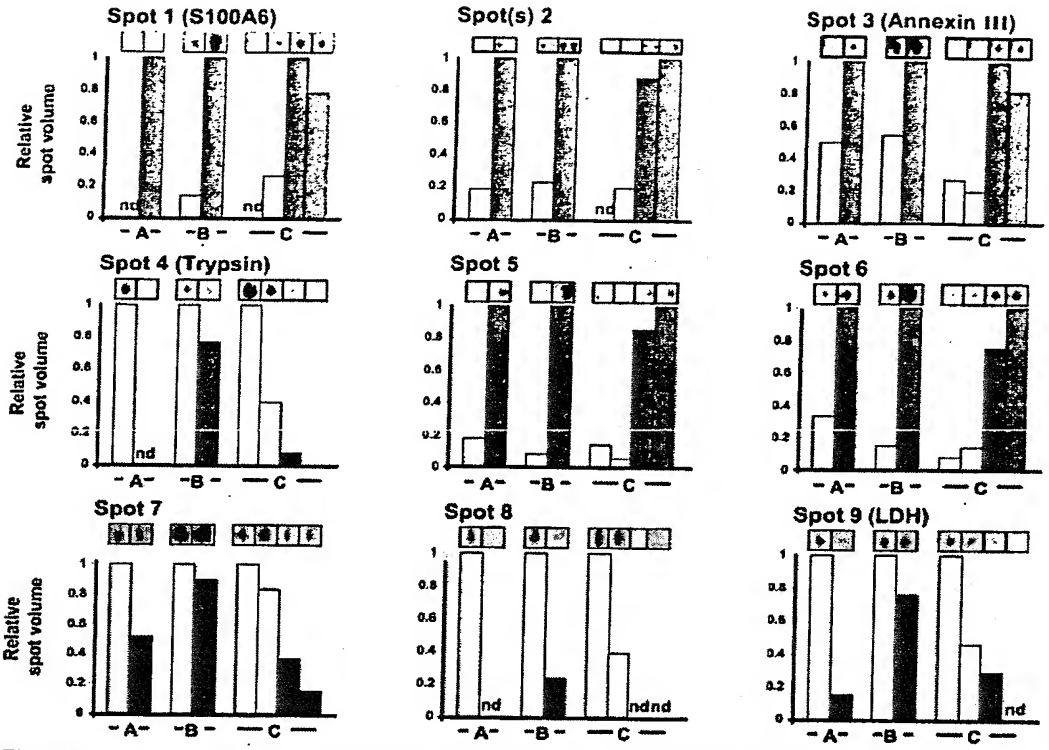

Figure 4. Graphs showing relative spot volumes from normal (white bars) and tumor (grey bars) samples for each of nine spots identified as differentially expressed. Each graph shows data from three experimental data sets A, B and C. Sets A and B are from patient-matched normal and tumor samples. Set C is from four unmatched patients. Within each set the relative spot volume for the most intense spot was arbitrarily set to one. Corresponding images of the respective spots from each of the gels used in the study are shown. Spot 2 appears either as a unique spot or as a doublet. Both spots of the doublet have been shown by MALDI-TOF to contain the same protein. Spot volumes for Spot 2 are presented where appropriate as the sum of the volumes for the two spots. nd = not detected; LDH = lactate dehydrogenase.

mes of each of the nine spots to be determined (Fig. 4). This analysis confirmed, for each spot, our visual assessment.

3.5 Strategy for the identification of proteins

Identification of proteins from 2-D gels of pancreatic tissue was undertaken by peptide mass fingerprinting using MALDI-TOF mass and MALDI-TOF/TOF based MS/MS analysis. The limited amounts of material available from silver-stained gels of laser-captured proteins are largely insufficient, in our hands, to routinely give reliable identifications by MALDI-TOF. Thus, proteins of interest on silver-stained gels of microdissected samples have been matched to the equivalent proteins spots from either silver stained or Coomassie blue stained gels prepared from large quantities of whole (*i.e.* undissected) tissue sections from either control tissue or tissue enriched in tumor. The latter spots have then been processed for MALDI-TOF MS or MS/MS. However, several factors need to be considered. There are substantial differences between spot profiles on microdissected and whole tissue sample gels (Fig. 2). Additionally, profiles differ markedly between silver and Coomassie blue stained gels. Thus, matching of spots on microdissected gels to the equivalent ones derived from whole sections is not a trivial task and even in cases where matches seem clear, caution must be exercised before assigning identification to a specific protein spot. Furthermore, in some cases MALDI-TOF data, even from proteins recovered from Coomassie blue stained gels, were not adequate to identify the proteins. Hence, in such cases, we have undertaken analysis using MS/MS by MALDI-TOF/TOF to confirm identifications.

One protein that was up-regulated in tumor samples was potentially 'identified' by MALDI-TOF as the calcium-binding protein, S100A6 (accession number NP_055439) although the score was below significance. MALDI-TOF/TOF analysis of this same digest produced MS data from which several MS/MS spectra were acquired (data not shown) such that the protein was identified as S100A6 with very high confidence (MASCOT score of 265). Using this approach a subset of differentially regulated proteins has been identified. These are annexin III (accession number NP_005130), lactate dehydrogenase (CAA68701) and trypsin (NP_002760). We are currently in the process of identifying the other differentially regulated proteins. In each case, however, independent validation of the changes in protein expression revealed by the 2-D gel analysis will be required.

3.6 Independent validation of the expression of S100A6

The expression of S100A6 in normal and malignant pancreatic cancer samples was examined immunohistochemically using a commercially available polyclonal antibody raised against recombinant human S100A6. The S100 family of proteins comprises 20 different family members [27] with 25–65% sequence homology. While the company who produced the antibody (DAKO) ruled out cross-reactivity with S100A1, S100A2, S100A4 and S100B, we were concerned that cross-reactivity with other family members could mislead our data analysis. We have identified, by MALDI-TOF, other S100 family members present on colloidal Coomassie blue stained 2-D gels containing proteins extracted from pancreas samples and are thus aware that they are present in our samples. We therefore carried out Western analysis on a pancreas tumor protein extract separated in two dimensions. A single spot, correctly positioned for S100A6 was observed (not shown). Thus no cross-reactivity with other S100 family members on our gels was detected. We then used this antibody for the detection of S100A6 on a tissue array comprising a series of normal and malignant pancreatic specimens on a single histological slide. Selected sections, showing a range of staining patterns in normal and tumor tissue, stained for S100A6 are shown in Fig. 5. Significantly, no S100A6 expression was detected in acinar cells or islet cells of the pancreas and there was only very occasional weak staining in stromal cells.

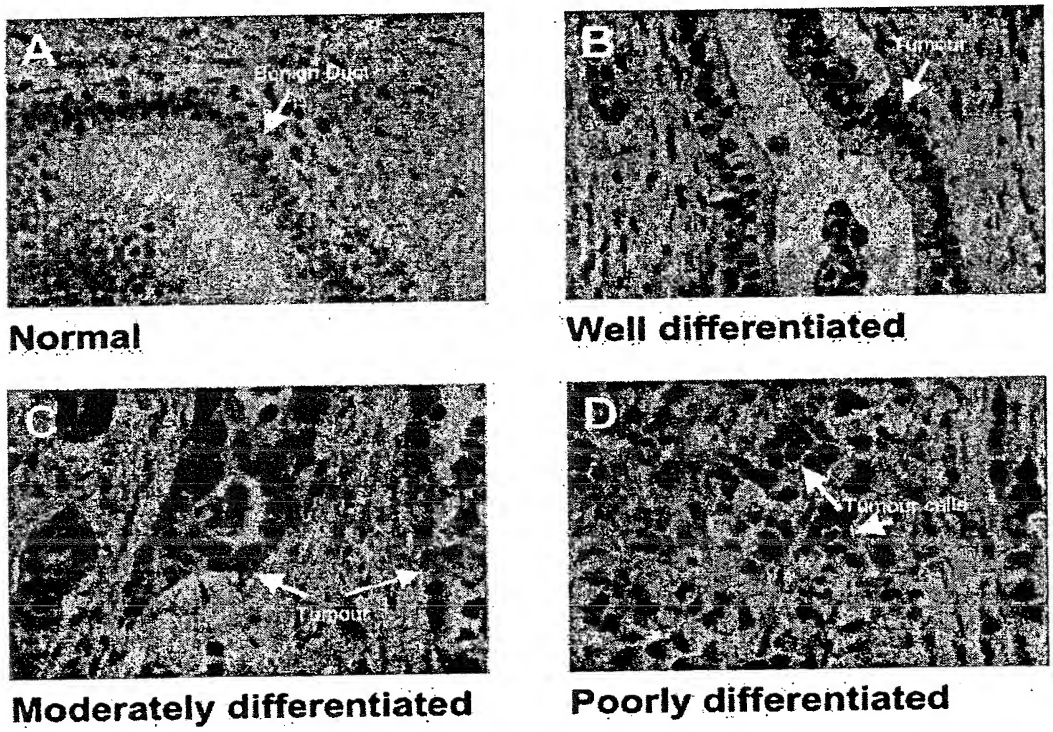

Figure 5. Photomicrographs showing selected sections from a pancreatic tissue array, immunohistochemically stained for S100A6.

Table 1. Summary of immunohistochemical data from the tissue array.

	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	Total	% C	% N
	−	−	−	+	+	−	+	+	+	++	++	++	+	+++	++	+++			
Normal	10		5				1		2								18	17.0	44.4
Well Diff.	5		1		1				3		1						11	45.4	45.4
Mod. Diff.	4				1				6		3		5		3		22	81.9	77.2
P.D.	2				1		1		4		1		1		1		11	81.9	72.2

Samples, categorised histologically into four distinct groups, normal, well differentiated tumor (well diff.), moderately differentiated tumor (mod. diff.), or poorly differentiated tumor (P.D.) are indicated, with the total number in each category shown. The degree of staining (− = negative; + = weak; ++ = intermediate; +++ = strong) in the cytoplasmic (C) and nuclear (N) compartment of the ductal epithelia, or tumor cells was determined and the number of cases in each category is shown. In addition, the percentage of samples in each category positive for S100A6 staining, regardless of the intensity of the staining, is given for the cytoplasmic and the nuclear compartments.

Since our 2-D gels contained proteins derived from normal or malignant ductal epithelial cells, we were particularly interested to determine whether these cells expressed S100A6. The samples on our tissue array fell into five histological categories: normal, pancreatitis (not reported in this study), well differentiated tumor, moderately differentiated tumor, or poorly differentiated tumor. The degree of staining in the cytoplasmic (C) and nuclear (N) compartment of the ductal epithelia, or tumor cells in each of the cases in these categories was assessed and the results are shown in Table I. Normal ductal epithelial cells were devoid of detectable S100A6 staining in 55% of cases (10/18). Moreover, in the eight cases where staining was detected, it was for the most part (5/8 cases), weak and in the nuclear compartment only. Of note, no normal ducts exhibited strong S100A6 staining. The pattern of S100A6 expression in well differentiated tumor cells was somewhat similar, with approximately half (5/11) of the cases lacking S100A6 staining and none of the cases displaying strong staining. Statistical analysis showed no evidence for a difference in either cytoplasmic or nuclear staining (Chi Square test: $p = 0.172$ and 0.139 respectively) in normal ductal epithelial cells *versus* cancer cells in well differentiated tumors.

The result was different however in the cases of moderately and poorly differentiated tumors. In both these categories, 81.8% of cases (18/22 and 9/11; moderately and poorly differentiated tumors respectively) showed cytoplasmic staining compared with only 17% of normal cases (3/18). These differences were found to be statistically significant (Chi square test: $p = 0.0002$ and 0.002 for moderately and poorly differentiated respectively compared to normal). Likewise, nuclear staining was elevated in these tumor categories compared to normal ductal epithelia. Moderately differentiated cases (77.3%, 17/22) and 72.7% of poorly differentiated (8/11) cases showed nuclear staining compared to 44.4% (8/18) of normal cases (Chi square test: $p = 0.0002$ and 0.023 for moderately and poorly differentiated tumors respectively compared to normal). Furthermore, a considerable proportion of moderately differentiated tumors (17/22) and poorly differentiated tumors (7/11) showed intermediate or strong nuclear staining compared with only 2/18 cases containing normal ducts. These data show that the profile of S100A6 expression in normal ducts is considerably different to that in moderately or poorly differentiated tumors.

4 Discussion

4.1 LCM facilitated analysis of protein profiles from pancreatic ductal epithelial cells

Despite much progress in uncovering the molecular mechanisms underlying pancreatic ductal adenocarcinoma, it remains a deadly disease with few therapeutic options. In this study we have examined whether a proteomic approach that investigated the differences in protein profiles between normal and malignant pancreatic samples could feasibly uncover proteins that are differentially expressed in these tissues. It is hoped that the identification of such proteins will enhance our understanding of this disease or provide early diagnostic or therapeutic markers for it. The choice of starting material was a critical one. PDAC accounts for 90% of all pancreatic malignancies and is the most aggressive malignancy of the pancreas. It arises in the pancreatic ducts and it is believed that the transformation of normal ductal cells is at the origin of this cancer. Comparisons between protein

profiles of undissected samples are of course possible. However, the numbers of normal and malignant cells in our samples vary substantially and are often small in comparison to the total cell population. Our data showed differences between undissected and dissected samples, particularly in the case of normal samples. This endorses the necessity to perform LCM on pancreas specimens as part of a study to uncover proteins expressed differentially between normal and malignant ductal epithelia and is supported by studies of other cancers [19–21]. We acknowledge however, the significance of the other cellular components of the pancreas. It is known, for instance, that stromal cells play an important role in the pathogenesis of pancreatic cancer and it is likely that protein expression profiles in stromal cells will increase our understanding of this disease.

4.2 Comparisons of equally loaded silver-stained gels showed consistent differences

Silver staining has a limited dynamic range and relatively poor linearity. However, its sensitivity is unmatched by any other currently available method. Quantitative comparisons between spot intensities on different gels would be much easier if a more linear staining system was available. We compared the fluorescent dye, SYPRO Ruby (Eugene, OR, USA) with silver stain for staining our 2-D gels. We found that SYPRO Ruby staining led to the detection of only a few protein spots (about 40–50) when similar amounts of protein to those available from laser captured samples were resolved (data not shown). This compared with approximately 800 spots detected with silver staining. Given the limited dynamic range and linearity of silver staining, it was important that comparisons were made between gels that have had equal amounts of loaded protein. The low concentrations of proteins available to us, combined with the presence of both a detergent and a reducing agent in the sample buffer rendered the use of common protein assays difficult. The method that we have used in the present work, based on SDS-PAGE, has enabled us to determine the relative protein concentrations in LCM derived samples against a reference sample and ensured that we had sufficient and equal amounts of normal and tumor samples for 2-DE separation and visualization. Comparison of the microdissected samples revealed that in general the nonmalignant and malignant protein profiles were very similar. Nonetheless, distinct differences were observed. Importantly, at least nine of these differences were conserved in four normal and four tumor samples. These included LDH and trypsin that were consistently less abundant in tumor cells than in normal cells, and annexin III and S100A6 that were consistently more abundant in tumor cells than in normal cells. While we have not yet further characterized the expression of trypsin, LDH and annexin III we have examined S100A6 expression in greater detail in pancreatic specimens.

4.3 S100A6 is overexpressed in pancreatic cancer cells

S100A6 is a low molecular mass (10 kDa) Ca^{2+} binding protein, the crystal structures of which (in the Ca^{2+}-free and Ca^{2+}-bound states) are consistent with it being a calcium sensor [28]. It belongs to a family of S100 proteins, members of which are expressed in a cell and tissue-specific manner and have been implicated in a variety of diseases, including cancer development [29] and metastasis [30]. It was first identified as a protein whose expression increased in human fibroblasts in response to growth factor stimulation [31]. More recently, its expression has been shown to increase in the renal collecting duct in response to vasopressin, suggesting a role in transepithelial ion transport [32]. It has also been implicated in the calcium dependent secretion of insulin from pancreatic beta cells [33] and most recently in the ubiquitinylation pathway regulating the proteolytic degradation of β-catenin [34]. Of relevance to this study, S100A6 is overexpressed in several tumors [31, 35, 36]. While its function in cancer is unknown, it has been linked to tumor progression [35, 36] and metastasis [35–37]. Our immunohistochemical analysis of a pancreas cancer tissue array revealed that the normal ductal cells in 83% of cases were devoid of detectable cytoplasmic S100A6, while over half lacked nuclear S100A6. In those normal ducts that exhibited S100A6 staining, the level was weak or intermediate. Well differentiated tumors showed slightly more cytoplasmic staining but similar nuclear staining. By contrast, moderately and poorly differentiated pancreatic cancers showed both a greater frequency and a higher intensity of S100A6 expression. It is important to note that the number of cases in the well differentiated and the poorly differentiated tumor groups was small. However, this pattern of staining potentially indicates that S100A6 may be involved in pancreatic cancer progression. The function of S100A6 and the mechanisms behind its overexpression in pancreatic cancer cells is unknown and must be the subject of future research. However, it indicates that our proteomic approach can uncover changes in protein expression that correlate with the malignant pancreatic phenotype. While this work was in progress, we have, using microarray technology, also identified S100A6 as highly overexpressed at the RNA level in pancreatic adenocarcinoma [38].

4.4 Alternative approaches may enable the discovery of further differentially expressed proteins

There are some important limitations to the combination of the LCM and 2-DE approach that we have taken. Obtaining pancreatic samples with sufficient normal pancreatic ductal cells was not trivial and could be restrictive. Moreover, the laser capture procedure is time consuming and laborious and yielded small quantities of protein for analysis. In effect, we were limited to studying 600–700 of the most highly abundant pancreatic ductal epithelial proteins that can be resolved by 2-DE (hydrophobic proteins are often poorly resolved). Despite these drawbacks, our study was informative. We have found several proteins whose expression was consistently altered between normal and malignant pancreatic ductal epithelial cells. Future developments will undoubtedly improve upon the sensitivity of 2-DE. Moreover, alternative proteomic approaches such as surface-enhanced laser desorption/ionisation mass spectrometry (SELDI-MS) or isotope-coded affinity tagging [39] need to be explored in pancreatic cancer research. SELDI-MS has already been successfully used to identify a potential protein biomarker for PDAC from pancreatic juice [40]. It is also suited for use in combination with LCM and could be used to complement a 2-DE approach to uncover differences between the malignant and nonmalignant cells that are not detected with 2-DE and *vice versa*.

5 Concluding remarks

In conclusion, we have shown that laser capture microdissection of pancreatic ductal epithelial cells can be used to generate sufficient protein of suitable quality for 2-DE based protein expression profiling. We were able to reproducibly detect changes in protein expression between nonmalignant and malignant pancreatic ductal epithelial cells. At present, nine such protein/spot changes have been detected. One of these proteins was identified by MS analysis as S100A6 and its expression was validated by an immunohistochemical approach, which showed a pattern of S100A6 expression in pancreatic cancer cells consistent with disease progression. The identification and analysis of other potentially differentially regulated proteins is ongoing, and preliminary data suggest that our 2-DE gels are indeed predictive of protein expression in normal and malignant pancreatic ductal epithelial cells. This indicates that LCM combined with 2-DE is a valuable strategy for the identification of proteins that are differentially expressed in pancreatic cancer. Moreover, the approach could be applied to the study of other pancreatic diseases such as pancreatitis or other tumor types.

We are grateful to R. Jenkins for help and advice with 2-DE based proteomics mass spectrometry. We thank A. Dodson, Department of Pathology, University of Liverpool for his assistance with immunohistochemistry. This work was supported by The Northwest Cancer Research Fund, The Royal Society, The Biotechnology and Biological Sciences Research Council, the Medical Research Council and Cancer Research UK.

Received February 14, 2003
Revised April 7, 2003
Accepted April 7, 2003

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